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(71) Applicant *(for all designated States except US)*: AKZO NOBEL N.V. [NL/NL]; Velperweg 76, NL–6824 BM Arnhem (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: NICOLSON, Lesley [GB/GB]; Flat 8, 65 Partickhill Road, Glasgow G11 5AD (GB). RIJKE, Eric, Onno [NL/NL]; De Haagbeuk 9, NL–5831 RR Boxmeer (NL).

(74) Agent: OGILVIE–EMANUELSON, Claudia, Maria; P.O. Box 20, NL–5340 BH Oss (NL).

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(54) Title: USE OF INTERLEUKIN–18 AS VACCINE ADJUVANT

(57) Abstract

The present invention relates to the use of interleukin–18 (IL–18) as a vaccine adjuvant, adjuvant compositions and vaccines comprising said IL–18, and various recombinant IL–18 to be used in said compositions and vaccines.

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USE OF INTERLEUKIN-18 AS VACCINE ADJUVANT

The present invention relates to the use of recombinant interleukin 18 (IL-18) as an adjuvant, adjuvant compositions and vaccines comprising said IL-18, and various recombinant IL-18 to be used in said compositions and vaccines.

Interleukin 18 (IL-18) is a novel cytokine, that can be isolated from the liver, and which is predominantly produced by activated marcophages. IL-18 has been reported to induce the production of interferon-γ (INF.-γ) in established Th1 cells, to stimulate NK cell cytotoxicity, and to activate the proliferation of Th1 but not Th2 cells (Okamura et al., Nature vol. 378:88 (1995); Stoll et al., J. Immunol. vol. 159 (1):298 (1997)). Additionally, IL-18 was also found to augment granulocyte-macrophage-CSF production, decrease IL-10 production but was not found to have an effect on IL-4 production by Con A-stimulated PBMC (Ushio et al., J.Immunol. vol. 156 (11):4274 (1996); Kohno et al., J Immunol. vol. 158(4):1541 (1997)).

Although these biological activities appear to be similar to those reported for IL-12, IL-18 exerts these effects independently from IL-12. These findings, and the fact that IL-18 differs structurally from IL-12, indicate that IL-18 and IL-12 are functionally distinct with respect to receptor binding and signal transduction pathways (Kohno et al., J. Immunol. 158(4):1541-1550, 1997). cDNA encoding murine and human IL-18 has been cloned (Okamura et al., Nature 378:88-91, 1995; Ushio et al., J. Immunol. 156(11):4271-4279, 1996). The gene encodes for a precursor protein which contains a leader sequence which resembles the IL-1 signature-like sequence. Although IL-18 and IL-1β proteins both contain this IL-1 signature-like sequences, the homology between the amino acid sequences of IL-18 and IL-1 is less than 20% and their biological activities were different in terms of induction of INF.-γ (Ushio, supra).

EP-A-712931 and EP-A- suggest the use of IL-18 as a therapeutic and/or prophylactic agent in case of INF.-γ-susceptive diseases such as AIDS, and condyloma acuminatum; malignant tumours such as renal cancer, granuloma, mycosis fungoides and cerebral tumour, articular

rheumatism and allergy. In addition IL-18 is suggested for use in so called "anti-tumour immunotherapy" using IL-2 to treat solid malignant tumours such as colonic cancer, rectal cancer, gastric cancer, thyroid carcinoma, cancer of the tongue, bladder carcinoma, choriocarcinoma, hepatoma, prostate cancer, carcinoma uteri, laryngeal lung cancer, breast cancer, malignant melanoma, Kaposi's sarcoma, cerebral tumour neuroblastoma, tumour of the ovary, testicular tumour, osteosarcoma, cancer of the pancreas, and others.

Vaccination against an infectious disease aims to elicit an immune response that limits clinical symptoms associated with infection by a pathogen. It is important that the correct type of immune reaction is triggered, since many types of immune mechanisms that can be activated are inadequate for control of the particular pathogen. Low responsiveness to vaccine antigens can be overcome by administering the antigens in combination with adjuvants. Adjuvants are defined as those components of a vaccine formulation other than the antigen which contribute to enhanced immune responsiveness to the antigen, e.g. aluminium salts, oil emulsions, derivatives of muramyl peptide, monophosphoryl lipid A, liposomes, QS21, MF-59, Iscoms, and the like.

The cellular and molecular mechanisms that are activated following vaccination are strongly influenced by the choice of adjuvant that is administered together with the vaccine antigen. Hence the selection of adjuvants may be as critical as the choice of vaccine antigens themselves in providing optimal efficacy.

It has now been surprisingly found that IL-18 has a potent adjuvant effect on the immune response of a subject to a vaccine. Because of this, IL-18 can be used as a vaccine adjuvant. Thus in one embodiment the invention provides for an adjuvant composition comprising an effective adjuvant amount of IL-18. The adjuvant composition comprising IL-18 can be administered concomitantly or sequentially with a vaccine formulation.

Alternatively, IL-18 can be included in the vaccine formulation. Thus in another embodiment the present invention provides for a vaccine comprising at least one active agent, an effective adjuvant amount of IL-18, and a pharmaceutical acceptable carrier or diluent. Preferably suitable for use as an adjuvant is an IL-18 that is closely related to the IL-18 naturally found in the subject animal or patient. Thus preferably the IL-18 is derived from the same species as the vaccine is designed for, e.g. canine IL-18 in the event of a vaccine for use in canines, human IL-18 in the event of a vaccine for use in humans, and so on. In a preferred embodiment, the Il-18 is derived from equine or canine for use in vaccination of dogs and horses respectively.

IL-18 according to the present invention can be the whole molecule or fragments thereof, provided said fragments have retained their adjuvanting ability. It should be understood that functional equivalents of IL-18 can also be used in the present invention. Functional equivalents are defined as modified IL-18 proteins which differ in amino acid sequence from wild type IL-18 but nevertheless have substantially the same adjuvanting activity as wild type IL-18. These modifications can constitute insertions, deletions, or conservative substitutions of one or more amino acids in the amino acid sequence of wild type IL-18. Also within the scope of the invention is an IL-18 molecule conjugated to another molecule, either direct or via the use of a conjugating agent (a linker), provided that said conjugation does not prevent or hinder the adjuvating effect of IL-18.

IL-18 of the present invention can be obtained via extraction or purification from natural sources, via organic chemical synthesis, or via recombinant DNA technology. Most preferred is the production of IL-18 via recombinant DNA technology. The recombinant production of Il-18 necessitates the use of genes or nucleotide sequences that encode said IL-18. IL-18 encoding nucleotide sequences have been published for murine, human and rat IL-18 respectively (Okamura, supra; Ushio, supra; B. Conti et al., J. Biol. Chem. 272 (4), pp. 2035-2037, 1997).

In a further embodiment the present invention provides for nucleotide sequences that code for IL-18, more especially canine and equine IL-18. The nucleotide sequences coding for canine IL-

18 and equine IL-18 are depicted in SEQ ID NO 1 and 3, respectively. The primary deduced amino acid structure of canine and equine IL 18 is given in SEQ ID NO 2 and 4, respectively.

The cloning of the nucleotide sequences encoding canine and equine IL-18, respectively, enables the production of pure IL-18, free from other cytokines. This is especcially useful in case of the production of IL-18-specific antibodies. These specific anti-IL-18-antibodies can be generated via techniques generally available. Preferably the specific anti-IL-18-antibodies are monoclonal anti-IL-18-antibodies. Thus the present invention furthermore provides for IL-18-specific antibodies, more particularly canine and/or equine IL-18-specific antibodies. The Il-18-specific antibodies according to the invention are suitable for use in diagnostics or for isolation and purification of IL-18 protein from crude preparations. Moreover, the antibodies can be used into develop assays for quantitative analysis of Il-18 production *in vitro* or for quantitaive measurements of IL-18 levels *in vivo*.

The adjuvant composition according to the present invention comprises IL-18 and a pharmaceutical acceptable carrier. Suitable pharmaceutical carriers are water, saline, and the like. Additionally, the adjuvant composition may comprises one or more other adjuvants such as oil emulsions, aluminium salts, derivatives of muramyl dipeptide, monophosphoryl lipid A, liposomes, QS21, MF-59, Iscoms, and the like. Preferably, Il-18 is used in conjunction with other cytokines such as for example IL-12. In a preferred embodiment, the adjuvant composition according to the invention comprises a DNA plasmid capable of expressing said IL-18. Said DNA plasmid comprises DNA sequences encoding IL-18 operably linked to transcriptional regulatory sequences. Nucleotide sequences encoding for other cytokines that are used in conjunction with IL-18 can be present on the same DNA plasmid or on a separate plasmid. Upon administration of such a DNA adjuvant composition to a subject, host cells take up and express encoded genes on the inoculated DNA, resulting in *in vivo* expression of said IL-18.

Vaccines according to the invention can be used for immunisation of humans and animals, such as for example swine, sheep, birds, cattle, dogs, cats, equines, fish and shell fish. A vaccine according to the invention comprises at least one active agent and an effective adjuvant amount of IL-18, i.e. an amount of IL-18 which will cause the vaccinated subject to produce an enhanced immunological response as compared to the vaccine without said IL-18.

The required effective amount of IL-18 in an adjuvant composition or vaccine according to the invention is dependent on the type of active agent used, the type of pathogen immunised against, as well as the type of vaccinated subject. Determination of the effective amount is well within the routine skills of the practitioner, and will generally be in the amount of 0.001 to 500 μg/dose. Preferably the amount will be between 0.01 and 50 μg/dose, more preferably 0.1 to 5μg/dose.

The active agent for use a vaccine according to the invention can be of viral, bacterial or parasitic origin. The active agent may either be the whole pathogen which causes the disease, or may consist of components derived from said pathogen. In the event the active agent is a whole pathogen, said pathogen may be a live pathogen or an inactivated pathogen. Live pathogens are considered to be either attenuated or naturally occurring mild strains of said pathogen. Inactivated pathogens are pathogens killed by chemical or physical means, that is, the inactivate or "killed" pathogen is no longer capable of replication. Suitable means for chemical inactivation are formaldehyde, glutaraldehyde, β-propiolactone, ethyleneimine and derivatives, and the like. Suitable means for physical inactivation are UV radiation, γ-radiation, "heat-shock", X-radiation, and the like. Alternatively, the active agent may constitute one or more components derived from said diseases causing pathogen, e.g. purified protein, protein-polysaccharide, protein-lipopolysaccharides, lipopolysaccharides, and the like.

In a preferred embodiment of the invention, the active agent is a DNA plasmid capable of *in vivo* expression of a pathogen or selected components derived from said pathogen. In addition, the

vaccine may comprise a DNA plasmid capable of expressing the IL-18 adjuvant *in vivo*. The DNA encoding said IL 18 adjuvant and the DNA encoding said pathogen or selected components may be present on one and the same plasmid, or may be present on separate plasmids. Upon administration of the DNA vaccine to a subject, host cells will take up and express *in vivo* said active agent as well as said IL-18. DNA vaccines are for example described in US 5,580,859.

Pharmaceutical acceptable carriers or diluents that can be used to formulate an adjuvant composition or a vaccine composition according to the invention are sterile and physiological compatible such as for example an aqueous buffer, a saline solution and the like. In addition stabilisers, preservatives and the like may be added to these compositions.

DNA plasmids that may be used in the adjuvant composition or vaccine according to the invention contain a carrier DNA fragment and a suitable expression cassette including transcriptional regulatory sequences, the target gene and other regulatory sequences, if desired. Examples of suitable plasmids include pBR322, pUC18 and pUC19, pNeo, pSVL, pMSG (commercially available from Pharmacia Biotech) and pMC1neo, pSG5, pXT1 and pBX (commercially available from Stratagene).

Examples of suitable transcriptional regulatory sequences comprise promoters such as the (human) cytomegalovirus immediate early promoter (Seed, B. et al., Nature 329, 840-842, 1987; Fynan, E.F. et al., PNAS 90, 11478-11482,1993; Ulmer, J.B. et al., Science 259, 1745-1748, 1993), Rous sarcoma virus LTR (RSV, Gorman, C.M. et al., PNAS 79, 6777-6781, 1982; Fynan et al., supra; Ulmer et al., supra), the MPSV LTR (Stacey et al., J. Virology 50, 725-732, 1984), SV40 immediate early promoter (Sprague J. et al., J. Virology 45, 773 ,1983), the metallothionein promoter (Brinster, R.L. et al., Nature 296, 39-42, 1982), the major late promoter of Ad2, the β-actin promoter (Tang et al., Nature 356, 152-154, 1992). Also suitable are the

The regulatory sequences may also include terminator and polyadenylation sequences. Amongst the sequences that can be used are the well known bovine growth hormone polyadenylation sequence, the SV40 polyadenylation sequence, the human cytomegalovirus (hCMV) terminator and polyadenylation sequences.

In principle, any transcriptional regulatory sequence can be used that is able to regulate the transcription of a gene in an eucaryotic cells as for example described in Sambrook et al, Molecular Cloning, a Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, 1989. In addition, the regulatory sequences may include an intron, for example hCMV intron A (Chapman, B.S. et al., Nucleic Acid Research 19, 3979-3986, 1991), the effect of which is to increase the expression of the encoded protein.

The compositions of the present invention may take any form that is suitable for oral or parenteral administration. For oral use, the adjuvant or vaccine compositions according to the invention may be formulated as solutions, syrups, suspensions, tablets, capsules and the like. For parenteral use, the compositions according to the present invention may be formulated in a form suitable for injection such as suspensions, solutions, dispersions, emulsions, and the like. Preparation of the compositions according to the present invention is carried out by means conventional for the skilled person.

Preferred administration routes are parenteral routes, e.g. intramuscular injection, intravenous injection, intradermal injection, subcutaneous injection, and mucosal routes, e.g. nasal drops, eye drops, (aerosol) sprays, and the like.

The following examples will illustrate the invention without limiting the invention thereto.

EXAMPLES

EXAMPLE 1

A. Recovery of alveolar macrophages

Lung macrophages were extracted from the lungs of a horse or dog (post mortem) by filling the lungs with tissue culture medium (equine - HBSS, canine RPMI) and recovering the medium and cells by pouring the fluid into centrifuge bottles (this procedure was performed several times to maximise recovery of cells from the lungs e.g. up to 3 litres of equine 'lung wash' was recovered). In the case of the dog this procedure was performed in a laminar flow hood to minimise the potential for bacterial contamination from the environment. Every effort was made to minimise contamination by red blood cells by gentle handling and avoidance of contamination from the external surface of the lung.

A.1 Canine

Cells recovered from the lung wash material by centrifugation of 'lung wash' at 1700 rpm for 10 minutes were resuspended in 20 ml medium (wash step), centrifuged at 1700 rpm for 10 minutes and the cell pellet resuspended in 20 ml or 40 ml medium depending on pellet size. Cell suspensions were transferred to tissue culture flasks; 10ml per $25cm^2$ flask. Flasks were gassed with CO_2 and incubated at 37°C for 4 hours to allow adherence of macrophages. The medium was then changed to remove non-adherent contaminating red blood cells and cultures incubated overnight at 37°C. Cultures were stimulated with PMA (5ng/ml) for 4 hours. Cells were recovered byremoving medium, washing with PBS then lysing cells as per Pharmacia Biotech Quick prep mRNA purification kit.

Medium:

Dulbecco's Modified Eagles Medium (Gibco cat number 31966-021) with added:

10% FCS
20 mM Hepes
100U/ml penicillin
100ug/ml streptomycin

A.2 Equine

Cells recovered from equine lungs in Hank's Buffered saline (HBSS) were spun down at 1800g for 10min at 4°C. The cell pellet was resuspended in HBSS and cells spun down at 1800g 10min at 4°C (wash step). This procedure was repeated. Cells were then washed a further twice using complete medium. The final cell pellet was resuspended in 5-10ml RPMI and a cell count performed. The cell suspension was diluted to $2x10^7$ cells/ml in complete medium (see below) and 10^8 cells (in 50ml medium) transferred to a $162cm^2$ flask. Cells were incubated overnight at 37°C in a 5% $C0_2$ atmosphere. Following overnight incubation the medium was removed and the monolayer washed twice with complete medium. 40ml of complete medium containing Lipopolysaccharide (LPS) at 10μg/ml was added to each flask and the cultures incubated for 6 hours (37°C, 5% $C0_2$). The medium was then removed and a cell scraper used to detach cells from the flask surface. Recovered cells were resuspended in 50ml of complete medium and pelleted at 200g for 5min. Pellets were resuspended in 50ml complete medium and centrifuged at 200g for 5 min (wash step). The supernatant was removed and the cells snap-frozen by immersion in dry-ice/100% ethanol. Frozen pellets were stored at -70°C.

Complete medium:
RPMI 1640
2% FCS
100U/ml penicillin
100ug/ml streptomycin

10mM Hepes buffer

2mM Glutamine

5×10^{-5} β-mercaptoethanol

B. Isolation of mRNA from stimulated cultures

mRNA was isolated using Pharmacia Quick Prep ® kit. Freshly recovered, or previously prepared cell pellets (the latter stored at -70°C) were used as the starting material for preparation of mRNA. mRNAs were prepared according to the manufacturer's protocols with minor modifications.

B.1 Canine

0.6ug mRNA was used as template for first strand cDNA synthesis using Pharmacia 1st strand cDNA synthesis kit according to manufacturer's instructions. The primer used was a NotI-d(T)18 bifunctional primer of sequence:

5'-AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTT-3' (SEQ ID NO 5)

as supplied in the kit. Total final volume of 1st strand cDNA reaction mix - 198ul

B.2 Equine

mRNA was treated with 10mM methylmercuryhydroxide - mRNA was resuspended in 20ul DEPC (diethylpyrocarbonate) treated water, incubated at 65°C for 5 mins and cooled to room temperature (RT). 2ul 100mM Methylmercuryhydroxide was added for 1 min at RT then 4ul 700mM beta-mercaptoethanol added at RT for 5'.

0.5ug treated mRNA was used as template for first strand cDNA synthesis using Pharmacia 1st strand cDNA synthesis kit according to manufacturer's instructions. The primer used was a NotI-d(T)18 bifunctional primer of sequence :

5'-AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTT-3' (SEQ ID NO 5) as supplied in the kit. Total final volume of 1st strand cDNA reaction mix - 86ul.

C. PCR reactions

Reaction mixes

template

*1x PCR reaction buffer

*200uM dNTP [equine PCR], 100uM dNTP [canine PCR]

*2mM $MgCl_2$

*2 units Amplitaq polymerase [equine PCR], 1.25 units [canine PCR]

50 pmol primer [equine PCR], 20 pmol [canine PCR]

* as supplied perkin Elmer Cetus kit :

primers supplied by - Cruachem, Glasgow

C.1 PCR reactions

PCR reactions using cDNA as target were performed. Some primer combinations produced no significant product after primary PCR and PCR products from this reaction were used in a secondary PCR reaction using identical or different primer conditions to that used in the primary PCR. 5ul cDNA was used in primary PCR's and I ul of primary PCR product in secondary PCRs. Annealing temperatures and cycling conditions were optimised for amplification of parts or whole equine and canine IL-18 cDNAs - examples of PCR reaction conditions are detailed below. PCR machine Perkin Elmer model 9600 was used for equine PCR reactions and Perkin Elmer Geneamp PCR system 2400 for canine reactions.

' = minutes; s = seconds

primary equine PCR reaction conditions: primers A+B, C+B, A+D, or C+D

94°C/5' (meaning 94°C for 5 minutes)

30 cycles of - 94°C/40s - 45°C/55s - 72°C/2' [all primer combinations]

72°C/ 7'

4°C until reaction tubes retrieved from PCR machine

Clonable product recovered from A+B and C+B reactions.

Secondary equine PCR reaction conditions: primers C+D

94°C/5'

30 cycles of - 94°C/45s - 45°C/1' - 72°C/2' [A+B reactions: template primary PCR A+B; C+D reactions: template primary PCR A+D or C+D]

72°C/7'

4°C until reaction tubes retrieved from PCR machine

Primary reactions were performed using A+D, A+B, or C+D then secondary PCR using priomers A+B or C+D to generate clonable A+B and C+D products

Primary canine PCR reaction conditions: primers A+E, A+B, A+D

94°C/5' before addition of enzyme ('hotstart') [A+E primer combination only]

95°C/5' before addition of enzyme ('hotstart') [A+D primer combination only]

30 cycles of 95°C/15s - 55 or 58°C/15s - 72°C/15s [A+D reactions]

30 cycles of 95°C/45s - 50°C/45s - 72°C/1' [A+B reactions]

30 cycles of 94°C/45s - 58°C/1' - 72°C/2' [A+E reactions]

72°C/60'

4°C until reaction tubes retrieved from PCR machine

Clonable products recovered A+E and A+B reactions only

Other canine IL-18 clones were recovered by secondary PCRs using primary PCRs with primer A in combination with primers B, D and E as target for secondary PCR using A+B, A+E and C+B.

Secondary canine PCR reactions:

94°/5' hotstsrt [optional]

30 cycles of 94°C/45s - 54 or 58°C/1' - 72°C/2' [A+B reactions: template primary PCR A+D]

30 cycles of 94°C/45s - 58 or 60°C/1' - 72°C/2' [A+E reactions: template primary PCR A+E]

30 cycles of 94°C/45s - 45°C/1' - 72°C/2' [C+B, C+D reactions: template primary PCR A+D]

72°C/7'

72°C/53°C [optional]

4°C until reaction tubes retrieced from PCR machine

C.2 Primers used

A (upstream): 5'-GCAGGAATAAAGATGGCTGC-3' (SEQ ID No 6)

B (downstream): 5'-GCGTTTTGAACAGTGAACAT-3' (SEQ ID No 7)

C (upstream): 5'-GACAATACGCTTTACTTTAT-3' (SEQ ID No 8)

D (downstream): 5'-GGCATGAAATTTTAATAGCTA-3' (SEQ ID No 9)

E (downstream) (used only for canine IL-18): 5'-GCTAGCTCTTGTTTTGAACAG-3' (SEQ ID No 10)

C.3 Derivation of consensus sequence

PCR reaction products from a minimum of three independent PCR reactions [primary and secondary] using the primer sets A+B, C+B, A+E (canine only), and C+D were cloned into Invitrogen TA cloning vector pCR2.1. IL-18 clones were sequenced using Amersham's ThermoSequenase cycle sequencing kit and LI-COR automated DNA sequencer model 4000L. and sequenced. Consensus sequences of the equine and canine IL-18 cDNAs were derived by

alignment of clones sequences using Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisc.

EXAMPLE 2

Groups of C57 BL/6 mice (female, 6 week-old) were injected intramuscularly (i.m) on day 0 in the upper leg muscle with a vaccine formulation containing as antigen inactivated Pseudorabies virus (PRV plus 0.1 LF tetanus toxoid (TT); $10^{6.7}$ $TCID_{50}$ per dose), . The antigen preparation was mixed together with 0.1 µg recombinant murine IL-18, expressed in E.coli (Pepro Tech, cat. no. 315-04) shortly, 1-2 hours before immunisation. In parallel groups of mice were immunised with vaccine antigen (PRV plus TT; $10^{6.7}$ $TCID_{50}$ per dose) in conjunction with saline as vehicle control. Four weeks after immunisation the animals were bled from the retroorbital plexus and their sera analysed for antigen-specific antibody titres using a method as described (Schijns et al., J. Immunol 153: 2029,1994)

One day after bleeding all groups of mice were challenged i.m. with virulent PRV (ADV phylaxia A25H, A-1015 1:600 diluted). Naïve unvaccinated animals all succumbed to the infection within 3-4 days. Among animals vaccinated with antigen only 30 % (3 out of 10) survived the infection, while among animals receiving the same amount of antigen together with only 0.1 µg IL-18 80 % (8 out of 10) survived the infection.

In addition, we observed that the levels of PRV specific antibodies were increased in the group of mice that received the antigen in conjunction with Il-18, when compared to animals vaccinated with antigen only (see table I)

Table I:

Vaccine	log PRV titre	log TT titre
None	6.3 ± 0.4	5.6 ± 0.5
Antigen only	7.5 ± 0.8	12.5 ± 0.8
Antigen + IL-18	9.1 ± 1.3	12.9 ± 0.6

LEGENDS

FIGURE 1: Survival rates of mice immunized with antigen and 0.1 µg IL-18 or antigen only after infection with virulent PRV.

CLAIMS:

1. A vaccine comprising an effective amount of IL-18.

2. IL-18 for use as a vaccine adjuvant.

3. The use of IL-18 for the manufacture of a pharmaceutical preparation for the vaccination of a subject.

4. An adjuvant composition or a DNA vaccine which comprises a DNA plasmid comprising a nucleotide sequence encoding an IL-18 protein, said nucleotide sequence being operably linked to transcriptional regulatory sequences, wherein said DNA plasmid is capable of *in vivo* expression of said IL-18 in the cells of the vaccinated subject.

5. IL-18 according to any of the claims 1 to 4 characterized in that said Il-18 is of the same origin as the subject to be vaccinated.

6. Il-18 according to any of the claims 1-5 characterized in that said IL-18 is canine or equine IL-18.

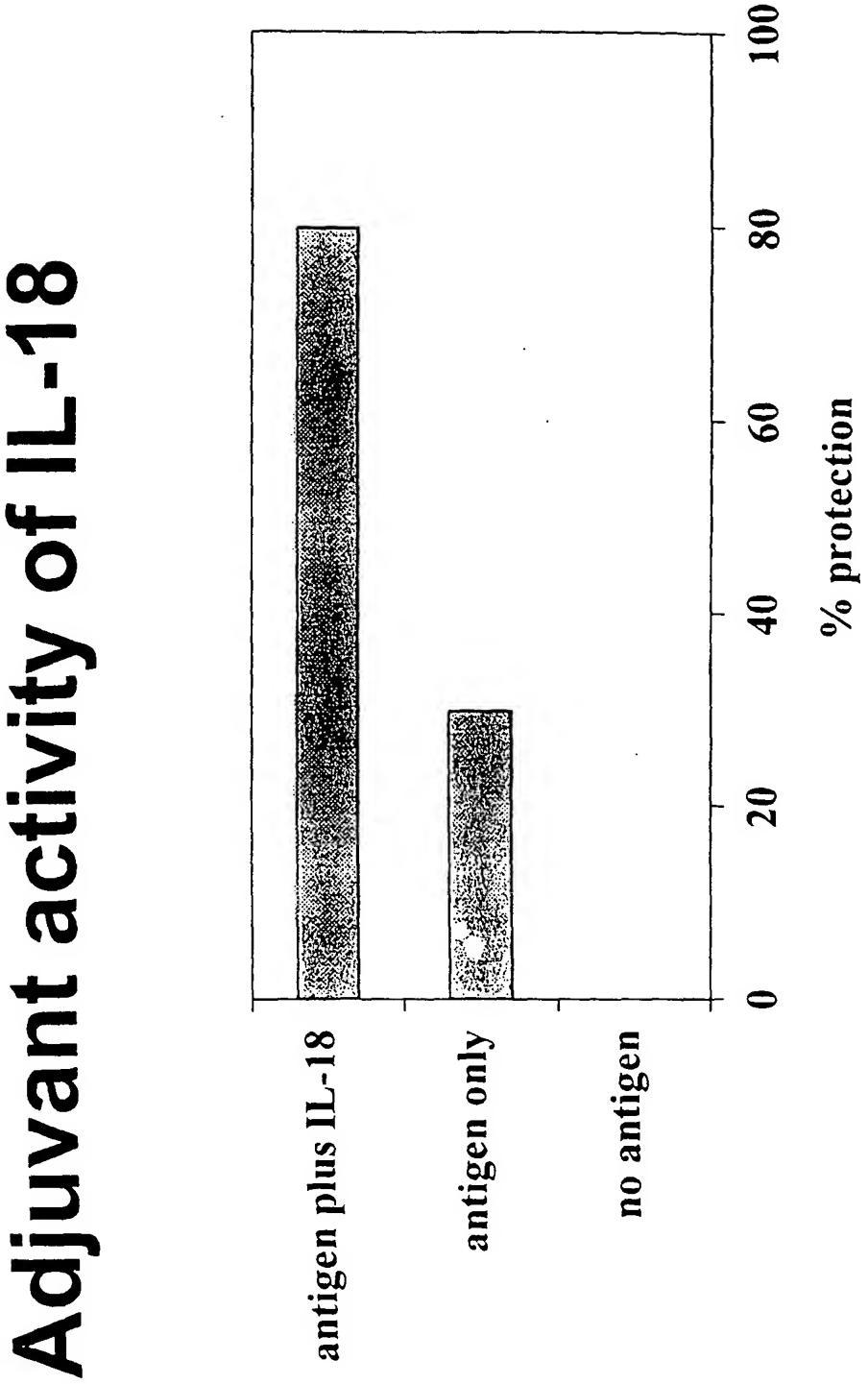
7. Protein having canine or equine IL-18 activity.

8. Protein according to claim 7 having the amino acid sequence depicted in SEQ ID NO:2 (canine IL-18) or SEQ ID NO:4 (equine IL-18).

9. Nucleotide sequence encoding a protein having canine or equine IL-18 activity.

10. Nucleotide sequence according to claim 9 which encodes for a protein having the amino acid sequence depicted in SEQ ID NO:2 or SEQ ID NO:4.

11. Nucleotide sequence depicted in SEQ ID NO:1 (canine IL-18) or SEQ ID NO:3 (equine IL-18).


Adjuvant activity of IL-18
antigen plus IL-18
antigen only
no antigen
0
20
40
60
80
100
% protection

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Akzo Nobel NV
(B) STREET: Velperweg 76
(C) CITY: Arnhem
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 6824 BM
(G) TELEPHONE: 0412 666379
(H) TELEFAX: 0412 650592

(ii) TITLE OF INVENTION: Use of Interleukin-18 as vaccine adjuvant

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGGCTGCTA ACCTAATAGA AGACAATTGC ATCAACCTTG TGAAAATGAA ATTTGTTAAC 60

AATACACTGT ACTTTAAAGC GGAAAGTGAT GAAGGCCTGG AATCAGATTA CTTTGGCAAG 120

CTTGAACCTA AACTCTCAAT CATACGAAAT TTGAACGACC AAGTCCTCTT CGTTAACGAG 180

GGAAATCAAC CTGTATTTGA GGATATGCCC GATTCTGACT GTACAGATAA TGCACCCCAT 240

ACCATATTTA TCATCTATAT GTATAAAGAT AGCCTCACTA GAGGTCTGGC AGTAACTATC 300

TCTGTGAAGT ATAAGACAAT GTCTACTCTC TCCTGTAAGA ACAAAACTAT TTCCTTTCAG 360

AAAATGAGTC CTCCGGATAG TATCAATGAT GAAGGAAATG ACATCATATT CTTTCAGAGA 420

AGTGTTCCAG GCCATGATGA TAAGATACAA TTTGAGTCTT CATTGTACAA AGGACACTTT 480

CTAGCTTGTA AAAAAGAGAA CGATCTTTTC AAACTCATTT TGAAAGACAA GGATGAAAAT 540

GGGGATAAAT CCATAATGTT CACTGTTCAA AACAAGAGCT AG 582

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 193 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ala Ala Asn Leu Ile Glu Asp Asn Cys Ile Asn Leu Val Lys Met
1 5 10 15

Lys Phe Val Asn Asn Thr Leu Tyr Phe Lys Ala Glu Ser Asp Glu Gly
20 25 30

Leu Glu Ser Asp Tyr Phe Gly Lys Leu Glu Pro Lys Leu Ser Ile Ile
35 40 45

Arg Asn Leu Asn Asp Gln Val Leu Phe Val Asn Glu Gly Asn Gln Pro
50 55 60

Val Phe Glu Asp Met Pro Asp Ser Asp Cys Thr Asp Asn Ala Pro His
65 70 75 80

Thr Ile Phe Ile Ile Tyr Met Tyr Lys Asp Ser Leu Thr Arg Gly Leu
85 90 95

Ala Val Thr Ile Ser Val Lys Tyr Lys Thr Met Ser Thr Leu Ser Cys
100 105 110

Lys Asn Lys Thr Ile Ser Phe Gln Lys Met Ser Pro Pro Asp Ser Ile
115 120 125

Asn Asp Glu Gly Asn Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly
130 135 140

His Asp Asp Lys Ile Gln Phe Glu Ser Ser Leu Tyr Lys Gly His Phe
145 150 155 160

Leu Ala Cys Lys Lys Glu Asn Asp Leu Phe Lys Leu Ile Leu Lys Asp
165 170 175

Lys Asp Glu Asn Gly Asp Lys Ser Ile Met Phe Thr Val Gln Asn Lys
180 185 190

Ser

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGGCTGCTG GACCAGTAGA AGACAATTGC ATTAGCTTGG TGGAAATGAA ATTTATTGAC 60

AACACACTTT ACTTTGTAGC TGAAAACGAT GAAAACCTGG AATCAGATTA CTTTGGCAGG 120

CTTGAACCTA AACTCTCAAT CATACGAAAT TTGAACGACC AAGTTCTCTT CATTAACCAG 180

GGAAATCAAC CTGTGTTTGA GGATATGCCT GATTCTGATT GTACAGACAA CGCACCCCAG 240

ACCGTATTTA TCATATATAT GTATAAAGAT AGCCTCACTA GAGGTCTAGC GGTAACCATC 300

TCTGTGAAGT GTGAGAAAAC GTCTACTCTC TCCTGTAAGA ACAAAATTAT TTCCTTTAAG 360

GAAATGAGTC CTCCTGAGAA TATCAATGAT GAAGGAAATG ACATCATATT CTTTCAGAGA 420

AGTGTTCCAG GACATGATGA TAAGATACAG TTTGAGTCTT CACTGTATAA AGGATACTTT 480

CTAGCTTGTG AAAAAGAGAA TGATCTTTTC AAACTCATTT TGAAAGAAAA GGATGAAAAT 540

GGGGATAAAT CTGTAATGTT CACTGTTCAA AACCAGAACT AG 582

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 193 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Ala Ala Gly Pro Val Glu Asp Asn Cys Ile Ser Leu Val Glu Met

```
1               5                   10                  15

Lys Phe Ile Asp Asn Thr Leu Tyr Phe Val Ala Glu Asn Asp Glu Asn
            20                  25                  30

Leu Glu Ser Asp Tyr Phe Gly Arg Leu Glu Pro Lys Leu Ser Ile Ile
        35                  40                  45

Arg Asn Leu Asn Asp Gln Val Leu Phe Ile Asn Gln Gly Asn Gln Pro
    50                  55                  60

Val Phe Glu Asp Met Pro Asp Ser Asp Cys Thr Asp Asn Ala Pro Gln
65                  70                  75                  80

Thr Val Phe Ile Ile Tyr Met Tyr Lys Asp Ser Leu Thr Arg Gly Leu
                85                  90                  95

Ala Val Thr Ile Ser Val Lys Cys Glu Lys Thr Ser Thr Leu Ser Cys
            100                 105                 110

Lys Asn Lys Ile Ile Ser Phe Lys Glu Met Ser Pro Pro Glu Asn Ile
        115                 120                 125

Asn Asp Glu Gly Asn Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly
    130                 135                 140

His Asp Asp Lys Ile Gln Phe Glu Ser Ser Leu Tyr Lys Gly Tyr Phe
145                 150                 155                 160

Leu Ala Cys Glu Lys Glu Asn Asp Leu Phe Lys Leu Ile Leu Lys Glu
                165                 170                 175

Lys Asp Glu Asn Gly Asp Lys Ser Val Met Phe Thr Val Gln Asn Gln
            180                 185                 190

Asn
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AACTGGAAGA ATTCGCGGCC GCAGGAATTT TTTTTTTTTT TTTTT 45

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCAGGAATAA AGATGGCTGC 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCGTTTTGAA CAGTGAACAT 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GACAATACGC TTTACTTTAT 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGCATGAAAT TTTAATAGCT A 21

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCTAGCTCTT GTTTTGAACA G 21

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/EP 99/03098

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39 A61K48/00 C07K14/54 C12N15/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 17799 A (THE TRUSTEES OF THEW UNIVERSITY OF PENSYLVANIA) 30 April 1998 (1998-04-30) page 107 -page 111 ---	1-5
X	TAN B. ET AL.: "Interferon-gamma-Inducing Factor Elicits Antitumor Immunity in Association with Interferon-gamma Production" JOURNAL OF IMMUNOTHERAPY, vol. 21, no. 1, January 1998 (1998-01), pages 48-55, XP002079489 the whole document --- -/--	1-5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 October 1999	21/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	STOLL S ET AL: "Production of IL-18 (IFN-gamma- inducing factor) messenger RNA and functional protein by murine keratinocytes." JOURNAL OF IMMUNOLOGY, (1997 JUL 1) 159 (1) 298-302, XP002079490 the whole document	1-11
P,A	USHIO S ET AL: "Cloning of the cDNA for human IFN-gamma- inducing factor, expression in Escherichia coli, and studies on the biologic activities of the protein." JOURNAL OF IMMUNOLOGY, (1996 JUN 1) 156 (11) 4274-9, XP002079491 the whole document	1-11
A	WO 98 10072 A (CORNELL RESEARCH FOUNDATION, INC.) 12 March 1998 (1998-03-12) the whole document	1-11
X	KIM J J ET AL: "Modulation of amplitude and direction of in vivo immune responses by co-administration of cytokine gene expression cassettes with DNA immunogens." EUROPEAN JOURNAL OF IMMUNOLOGY, (1998 MAR) 28 (3) 1089-103. , XP002118496 the whole document	1-6
P,X	OKANO F ET AL: "Cloning of cDNA for canine interleukin - 18 and canine interleukin-1beta converting enzyme and expression of canine interleukin - 18." JOURNAL OF INTERFERON AND CYTOKINE RESEARCH, (1999 JAN) 19 (1) 27-32. , XP002118497 the whole document	2,7-11
P,X	DATABASE WPI Section Ch, Week 199914 Derwent Publications Ltd., London, GB; Class B04, AN 1999-167427 XP002118498 & WO 99 07851 A (TORAY IND INC), 18 February 1999 (1999-02-18) abstract	2,7-11

INTERNATIONAL SEARCH REPORT

nformation on patent family members

International Application No
PCT/EP 99/03098

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9817799 A	30-04-1998	AU 5002297 A	15-05-1998
WO 9810072 A	12-03-1998	AU 4260497 A	26-03-1998
WO 9907851 A	18-02-1999	AU 8561198 A	01-03-1999